

EVALUATION OF TECHNIQUES FOR AGE DETERMINATION OF FRESHWATER MUSSELS (UNIONIDAE)

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ABSTRACT

Age validation and an assessment of four age determination techniques; shell ashing, thin-sectioning, acetate peels, and enumeration of external growth bands, were conducted on several species of freshwater mussels (Unionidae) in southwestern Virginia. The recovery of tagged and marked specimens of four species after one to three years confirmed the formation of one distinct annulus per year on and in shells. Thin-sectioning of valves was the most effective technique for aging and provided a high degree of both accuracy and precision. Shell ashing was totally unreliable, and acetate peels were inferior to thin-sections. The commonly used method of counting external growth bands on shells consistently underestimated the ages of older specimens and is of limited use in age determination of unionids.

The determination of absolute ages of bivalves is essential to derive population statistics for managing their harvest and conservation. Shells (valves) of freshwater mussels (Unionidae) exhibit pronounced bands or rings on their external surface, and the distance between bands decreases progressively with an increase in shell size. The significance of these bands and their use to derive absolute ages of mussels was discussed by early researchers (LeFevre and Curtis, 1912; Isley, 1914; Coker *et al.*, 1921). Based on the cyclical periodicity of band formation on valves, ages of freshwater mussels have been determined using the techniques of enumerating growth rings on the valve surface (Chamberlain, 1931; Stansbery, 1961), and ashing shells in a muffle furnace to separate the bands (Sterrett and Saville, 1975). The occurrence of growth bands within radial cross-sections of the shell and hinge ligament has provided an additional means of age determination (Hendelberg, 1960; Bjork, 1962; Ray, 1978; McCuaig and Green, 1983).

In most early attempts to age unionids, investigators relied on the visibility of growth bands on the outer surface of shells. Although these bands can be used to delimit age of some species, in other species subjective and conflicting

ages typically result. Growth bands on lentic species, which grow rapidly early in life, are characterized by regular spacing and distinctness (Chamberlain, 1931; Stansbery, 1961), whereas those on stream-dwelling mussels are less pronounced (Grier, 1922; Brown *et al.*, 1938). Investigations to determine age from external growth bands of riverine mussels, hereafter called the growth ring method, is often hampered by erosion of the shell surface, obscurity of bands on dark-colored valves, subjectivity in distinguishing annuli from stress-produced checks, and the inability to count closely deposited bands near the valve margin of older specimens (Ansell, 1968; Coon *et al.*, 1977; Lutz and Rhoads, 1980). Population statistics derived from this method, which apparently lacks both accuracy and precision, are therefore fraught with problems.

In contrast to the growth ring method most often used on freshwater bivalves, the techniques for determining ages of marine bivalves have been rigorously tested and are apparently more reliable. Most age studies of marine bivalves since Barker (1964) have used two sectioning techniques, thin-sections or acetate peels, to determine absolute ages; these methods are now used routinely in marine malacology (Clark, 1980). Both the chondrophore and entire valve of marine clams have proven to be useful for age determinations (Ropes and O'Brien, 1980), and detailed descriptions of the methods

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are provided by Lutz and Rhoads (1980) and Ropes (1984).

The annual formation of winter growth bands on the valve surface of some freshwater mussel species has been documented (Isley, 1914; Chamberlain, 1931; Negus, 1966; Haukioja and Hakala, 1978), but the formation of internal annuli lacks appropriate verification. Most studies that have estimated ages of mussels by these various methods typically omit age validation (i.e. proof of the accuracy of the technique). Validation of these methods for mussels is necessary because of the presence of less prominent, stress-related growth checks in bivalve shells, termed pseudoannuli or "false" annuli. Some researchers have been able to distinguish the difference between annuli and "false" annuli with relative ease (Chamberlain, 1931; Negus, 1966; Day, 1984); others have had difficulty, especially with riverine species (Coon *et al.*, 1977; Haukioja and Hakala, 1978). Previous studies with unionids in the upper Tennessee River drainage, of Virginia and Tennessee, have also experienced difficulty in delimiting annuli and recognized the need for validation (Zale, 1980; Weaver, 1981). Age validation is an essential prerequisite for obtaining sound population statistics, and the application of routine but unvalidated methods to all species can result in significant misinterpretations of biological data (Beamish and McFarlane, 1983a, 1983b).

The three objectives of our study were: (1) validation of the annual formation of growth bands on and in the valves of various sizes and species of unionid mussels; (2) tests of the utility of shell ashing, thin-sectioning, and acetate peels for freshwater mussels; (3) comparison of the ages of specimens derived from the growth ring and thin-sectioning methods.

MATERIALS AND METHODS

ANNULUS VARIATION

A mark and recovery program was conducted from 1979 to 1983 to validate the annual deposition of growth bands, to determine the season of annulus formation, and to provide empirical data on mussel growth. Four relatively common mussel species, representing three subfamilies of unionids, were selected for this phase of the study: *Pleurobema oviforme* (Conrad, 1834); *Lasmigona subviridis* (Conrad, 1835); *Villosa vanuxemi* (Lea, 1838); and *Medionidus conradicus* (Lea, 1834). Specimens were obtained from three sites in western Virginia: New River, Montgomery County; North Fork Holston River, Smyth County; Big Moccasin Creek, Russell County. A total of 1452 adult mussels were collected by hand, transported to our laboratory, and held in a 300 l aerated, recirculating tank (Table 1). Each specimen was measured (length and height) with calipers to the nearest 0.1 mm and marked by one of three methods, numbered tag only, tag plus valve notch, and tag plus painted valve. These marking methods were used to record shell growth for a known time period and to recognize differences between annuli and other bands (false annuli) formed externally and internally on the valves.

One valve of each mussel was tagged with a 3 x 5 mm fluorescent orange, sequentially numbered disc tag (Floy Tag Company, Seattle, Washington), held in place by Duro

superglue (Loctite Corporation, Cleveland, Ohio). A small triangular notch was filed in the ventral margin of notched specimens, and red fingernail polish was applied to the shell margins of painted specimens. The marked specimens were transplanted to two sites (I and II) in each stream; specimens at site I (15 to 25 m² in area) were tagged and 7% were painted, and those at site II (0.7 to 3 m²) were tagged and notched (Table 1). Mussels were returned to their collection sites within 2 weeks and placed, properly oriented, in the substratum. At site I in the New River, 150 tagged mussels were divided among three substrata-filled chicken wire enclosures (13 mm mesh; 76 x 76 x 13 cm) set into the substratum to inhibit mussel dispersal and facilitate periodic examination. The remaining mussels at this site were placed near the enclosures. Sites in all three streams were identified either by landmarks, streambed features, or markers.

In each stream, mussels at site I were recovered after 1 year for annulus validation, and a sample of about 12 mussels at site II was collected quarterly during the first year for examination of seasonality in growth band deposition. Some specimens that could not be found 1 year after planting were collected up to 4 years later (1983). Recovered mussels were sacrificed, and incremental growth on valves was measured and examined for annulus formation externally and internally, under a dissecting microscope.

EVALUATION OF AGE TECHNIQUES

Ashing of shells to separate growth layers followed procedures similar to those used by Sterrett and Saville (1974). Initial cuts made on a Buehler Isomet low-speed saw unit with a diamond-impregnated blade (Buehler Ltd., Evanston, Illinois) were: (1) from the umbo to the shell margin along the vector of maximum length, and (2) from the umbo to the shell margin perpendicular to the first cut. The triangular wedges of shell produced by these cuts, with sectioned surface exposed on two sides, were ashed in a muffle furnace. Sterrett and Saville (1974) recommended ashing at either 500°C for 10 minutes or 600°C for 5 minutes. Because temperature and time are the factors apparently crucial for producing good results, a size range of shells (20-80 mm) was ashed at both of the recommended times and temperatures. However, the resulting ashed shells were too brittle to allow effective separation of many of the growth layers. Therefore, we conducted a series of ashing time and temperature trials to evaluate the utility of this technique: 300°C for 1, 5, 10, 15, or 20 min; 400°C for 1, 5, 10, or 15 min; 500°C for 1, 5, or 10 min; and 600°C for 1 or 5 min. Preliminary ashing tests indicated that each of these combinations of times and temperatures could produce usable results. Three shells, small (< 40 mm), medium (40-60 mm), and large (> 60 mm), were ashed in each of the 14 trials. All trials were later replicated to corroborate initial results. Utility of the shell ashing technique was assessed by (1) how well annual layers could be separated, and (2) how well growth bands could be distinguished externally and in cross-section.

Thin-sectioning of valves followed procedures similar to those described by Clark (1980), in which a low speed saw unit and diamond-impregnated blade was used. An initial

Table 1. Number of mussels of four species tagged in 1979 - 1982 at two sites each on Big Moccasin Creek (BMC), North Fork Holston River (NFHR), and New River (NR), western Virginia.

Stream, Site and Date	<i>Pleurobema oviforme</i>	<i>Medionidus conradicus</i>	<i>Villosa vanuxemi</i>	<i>Lasmigona subviridis</i>	Total
BMC I					
Oct 1979	—	63	29	—	92
Oct 1980	39	2	6	—	47
Sept 1981	101	165	103	—	369
BMC II					
Jul 1982	2	35	12	—	49
NFHR I					
Sept 1981	152	139	108	—	399
NFHR II					
Jul 1982	30	27	41	—	98
NR I					
Apr 1982	—	—	—	320	320
NR II					
Jul 1982	—	—	—	78	78
Total	324	431	299	398	1452

cross-sectional cut from the umbo to the shell margin followed the vector of maximum growth (postero-ventrally), since it generally intersected growth lines at right angles. Shell cuts were then bonded to petrographic micro-slides (27 x 46 mm) with epoxy glue (Buehler epo-quick) and vacuum-sealed into a petrographic chuck attached to the cutting arm of the saw. Because the thickness of the second cut was critical to producing thin-sections of suitable quality, several cuts ranging from 200 to 380 μm were made to determine optimal thickness for growth band detection. A thickness of 280 μm was considered to be best for consistent, high resolution thin-sections and was used in all subsequent sectioning of valves.

The utility of thin-sectioning was evaluated on a variety of mussel species from rivers in southwestern Virginia. Shell lengths ranged from 15 mm for *Medionidus conradicus* to 210 mm for *Potamilus alatus* (Say, 1817), although most shells were 20 to 80 mm long. Shells longer than 60 mm had to be cut more than once because the saw blade was only 114 mm in diameter. The final cut through the umbonal region of large shells included all internal growth lines. Sectioned shells and derived thin-sections were examined under 4X magnification, and felt-tip pen marks were made adjacent to the point where each growth line exited at the shell surface. The cross-sectioned shell was then superimposed on the marked thin-sections. This juxtaposition of shells allowed for visual comparison of internal with external growth lines to corroborate contiguity and to identify false annuli on the valves.

Acetate peels from sectioned shells followed the method of Kennish *et al.* (1980). Shells of *Pleurobema oviforme*, *Medionidus conradicus*, *Villosa vanuxemi*, as well as *Fusconaia cor* (Conrad, 1834) and *F. cuneolus* (Lea, 1840), two federally endangered species, were separated into small (< 40 mm), medium (40-60 mm), and large size groups (> 60 mm). An initial cross-sectional cut was made with the low-speed saw from the umbo to the shell margin along the vec-

tor of maximum growth. Although Kennish *et al.* (1980) suggested pre-embedding the valves in an epoxy resin first to prevent fracturing during sectioning, the stability of the low-speed saw allowed sectioning of most shells without fracture (Clark, 1980). Valve sections were then ground by hand on sequentially finer grit sizes: 320, 400, and 600 (Buehler carborundum grits) and polished with polishing alumina (Fisher Scientific Co., Fairlawn, New Jersey) on felt polishing cloth. Because acid-etching is the critical step in this technique and is apparently related to shell structure, mineralogy, organic content, and state of preservation (Kennish *et al.*, 1980), etching times and HCl concentrations are expected to differ slightly among species. Therefore, polished sections of each species and size group were etched in a dilute solution of HCl at various concentrations (1%, 5%, 10%) and time periods (15 sec to 5 min). This allowed development of an optimal procedure for shells of a given size and species. One valve was used in each of the etching time and HCl concentration trials. The etched shell sections were washed under running water and dried.

In the last step of the peel process, we placed the etched section firmly on a strip of acetate (2 mm thick) covered with acetone, and pressed for 30 sec. After the acetone dried completely (2-3 hr), the valve was pulled from the acetate strip, leaving an imprint (the peel) on the acetate. Internal growth bands on the peel were counted under 4 to 10X magnification. Quality of the acetate peels was judged by two criteria: clarity of growth bands in the umbonal region, and degree to which bands could be traced from the umbo to the shell margin.

COMPARISON OF EXTERNAL AND INTERNAL AGES

The valves of 82 specimens of *Fusconaia cor* and *Pleurobema oviforme* were selected for this comparison.

These species had relatively distinct external growth bands and were aged by the growth ring method. Later, the same valves were thin-sectioned, as previously described. Ages determined by these two methods were plotted graphically, and a Wilcoxon signed rank test was used to compare differences.

RESULTS

ANNULUS VALIDATION

A total of 521 (36%) of the 1452 marked mussels was recovered from the three streams (Table 2). Recovery rates of specimens from Big Moccasin Creek and the North Fork Holston River were similar, 49.1 and 47.1% respectively; the largest species, *Pleurobema oviforme*, was the most frequently recovered. Both sites on the New River yielded low returns (3.2%) because of specific problems. Muskrats (*Ondatra zibethicus* L. along the New River removed 55 marked specimens (found in shell middens) in June-July 1982, and one enclosure of 50 mussels was vandalized in October. In addition, a thick mat of *Elodea* developed by fall 1982 and summer 1983, and caused considerable siltation and mortality of marked mussels.

Of the three marking methods tested, notching of



Fig. 1. Thin-section of the umbonal region of *Pleurobema oviforme* showing internal growth lines (bar = 1 mm).

Table 2. Recovery and validation of annulus formation on mussels marked in Big Moccasin Creek (BMC), North Fork Holston River (NFHR), and New River (NR), western Virginia.

Stream/Species	Mussels Recovered		No. validated
	No.	%	
Big Moccasin Creek			
<i>Pleurobema oviforme</i>	83	58	7
<i>Medionidus conradicus</i>	101	38	10
<i>Villosa vanuxemi</i>	90	60	9
Subtotal	274	49	26
North Fork Holston River			
<i>P. oviforme</i>	109	60	4
<i>M. conradicus</i>	63	38	9
<i>V. vanuxemi</i>	62	42	20
Subtotal	234	47	33
New River			
<i>Lasmigona subviridis</i>	13	3	4
Total	521		63

valves was the most useful for recording shell growth and annulus deposition. Annuli appeared as dark bands in sectioned valves (Clark, 1974; Lutz and Rhoads, 1980), and were evident on 25 (27%) of the 94 notched specimens recovered at site II in the streams. Notching readily identified the origin of incremental growth and subsequent growth at the shell margin (Fig. 1). Thin-sections through the notch clearly delineated incremental growth and the presence of a growth band. An annulus was validated on all notched shells that grew more than 1 mm/yr and on several shells that grew 0.5 to 1.0 mm/yr. Several specimens, marked between 1979 and 1982 and collected in 1983, showed one annulus for each year at large.

Although the disc tags remained firmly attached to all specimens upon recovery, mussels with only tags were less useful for documenting growth bands. Only 38 (9%) of 425 recovered specimens from site I in the streams were useful for annulus validation. All mussels that grew more than 1.5 mm/yr were validated, but lack of precision with caliper measurements and a fragile shell margin prevented annulus validation on a higher percentage of the slower-growing specimens. Fingernail polish on shell margins was completely ineffective. Within 3 months after marking, it had sloughed from the shells apparently due to abrasion in the substratum.

Annulus formation was documented on 63 (12%) of the 521 specimens recovered from all sites (Table 2). Although this percentage appears low, only specimens with readily measurable incremental growth in length (1.0-1.5 mm, depending on marking method and species) could be used for validation. Occurrence of single (annual) growth bands was confirmed in the shells of all four marked species. Because 83% of the recovered specimens grew less than 1 mm, growth bands formed during the last year on these mussels were nearly indistinguishable from those formed during the penultimate year (Table 3). Growth was most rapid in *Lasmigona subviridis*, the most thin-shelled species, whereas

Table 3. Annual growth increments on mussels tagged and recovered in Big Moccasin Creek, North Fork Holston River, and New River, western Virginia.

Stream and Species	Annual increment (mm)									
	(0-<1)		(1-<2)		(2-<3)		(3-<4)		(4-<5)	
	No.	%	No.	%	No.	%	No.	%	No.	%
Big Moccasin Creek										
<i>Pleurobema oviforme</i>	67	81	12	15	2	2	1	1	1	1
<i>Medionidus conradicus</i>	92	91	6	6	2	3	—	—	—	—
<i>Villosa vanuxemi</i>	71	79	18	20	1	1	—	—	—	—
North Fork Holston River										
<i>P. oviforme</i>	98	90	11	10	—	—	—	—	—	—
<i>M. conradicus</i>	61	97	2	3	—	—	—	—	—	—
<i>V. vanuxemi</i>	51	82	11	18	—	—	—	—	—	—
New River										
<i>Lasmigona subviridis</i>	42	57	20	27	9	13	2	2	1	1
Total	482	83	80	13	14	3	3	<1	2	<1

adults of the other species grew more slowly.

Despite the slow growth of most of the recovered mussels, validation results provided convincing evidence of the formation of a single growth band each year. An annulus was formed in all tagged specimens that grew more than 1.5 mm and all tagged and notched specimens that grew more than 1.0 mm during the year. None of these lacked an annulus, nor had they more than one prominent growth band.

Only limited evidence was obtained on the seasonality of annulus formation, primarily because growth was slow throughout the year. Some specimens from the three streams provided evidence that the growth band had formed between January and May. No annulus was observed on specimens examined during fall and winter, but valves of four mussels examined in May and all of 16 valves examined in July had an annulus within the outer layer of incremental growth. Although sample sizes are small, it appears that the annulus is formed (becomes visible) in spring in western Virginia.

EVALUATION OF AGE TECHNIQUES

All ashing trials failed to meet our two criteria for suitability in age determination; i.e. separation of each annulus and recognition of growth bands externally and internally. Shells were either too brittle or inseparable at many annuli after the tests. Most shells ashed at 400°C for 10 and 15 min did separate along the first one to four annual growth bands. However, subsequent annuli could not be separated consistently; shells were brittle and crumbled when manipulated. Ashing also tended to obliterate the recognition of growth bands, making true annuli and false annuli indistinguishable.

The acetate peel technique was less effective than thin-sectioning, both in terms of clarity of growth bands in the umbo region and degree to which bands could be traced throughout the shell. Because of the similarity of the thin-section and peel techniques, and higher resolution produced by thin-sectioning, acetate peels produced by the method described were judged to be inferior to thin-sections for determining ages of mussel shells.

Thin-sectioning of valves was the most effective technique and usually provided a high degree of precision (Fig. 1). A section thickness of 280 μm produced consistent, high quality preparations for valves of all species over a wide range of shell lengths (15-210 mm). Ages of sectioned shells ranged from 3 to 56 years. The clarity of thin-sections resulted in a high degree of accuracy because the contrast between true annuli and false annuli was pronounced, and annuli could usually be traced continuously from the umbo to the shell margin. The entire sectioning procedure required 0.5 to 1 hour per valve (excluding overnight hardening of the epoxy glue), depending on shell size and thickness.

RECOGNITION OF ANNULI

Species that displayed distinct external annuli also had distinct internal annuli. Shells of *Pleurobema oviforme* and *Fusconaia cor* typically had well-defined internal and external annuli, unlike those of *F. cuneolus*, *Medionidus conradicus* and *Lasmigona subviridis*. Internal annuli of *Villosa vanuxemi* were readily distinguished, but the external growth bands were obscured by the dark periostracum of this species. Significant variability in the clarity of external annuli was also evident within a species; erosion of the shell surface was the major contributing factor, and this problem was directly correlated with age. Young specimens (3-6 yrs) were rarely affected, but in older individuals (7-15 yrs), the first and often second annulus was eroded. The first two annuli were typically missing in the oldest specimens (>15 yrs), and those older than 20 years could not be aged externally because the periostracum had become extensively damaged. Shell corrosion (dissolution) was also evident on shells from all three streams. Prior dissolution of calcium carbonate in the umbo region apparently resulted in pit formation.

False annuli occurred occasionally in all species examined. Thin-sectioning provided the best method for identifying false annuli because true annuli could be traced from umbo to shell margin. In contrast, false annuli were characterized by an incomplete growth line in thin sections (Fig. 2). Recognition of false annuli was much more difficult



Fig. 2. Thin-section of a valve of *Pleurobema oviforme* with a false annulus (arrow) among true annuli (bar = 0.5 mm).

on the shell surface. For example, the inclusion of small particles from the substratum into shells often caused the formation of a false annulus. This false growth check was observed most commonly in shells of females, particularly in *Villosa vanuxemi* from Big Moccasin Creek and the North Fork Holston River. Incorporation of these particles in the shell produced a thick, dark line both internally and externally on the shell (Fig. 3). This growth check appeared to be a true annulus on the shell surface, but was not continuous in the cross-sectioned shell.

EXTERNAL VERSUS INTERNAL AGES

Growth bands on the external surface of valves of *Pleurobema oviforme* and *Fusconaia cor* were readily visible and were more distinct than those in most other species available for such a comparison. Annuli were easily discerned on specimens 3 to 8 years old, but became more tightly grouped and less distinct on valves of mussels 8 to 15 years old. Shells of mussels more than 15 years old were difficult



Fig. 3. Thin-section of a valve of *Villosa vanuxemi* with the incorporation of sediment (arrow) into the valve (bar = 1 mm).

to age because surface annuli were nearly contiguous or indistinguishable even under magnification. If the periostracum was damaged by erosion or corrosion on older specimens, frequently no age estimates were possible. Erosion of valves was especially prevalent on old specimens of *P. oviforme*. No valves older than 20 years, as determined by the thin-section method, could be aged by the growth ring method because of periostracum damage. Erosion was also the probable cause for loss of the first and often second annulus on some valves older than age 6 years. The thin, organic-rich growth checks apparently were less solid than the calcium carbonate deposition in annual growth, and shell fractures in young specimens were occasionally evident along the annulus. However, cleavage lines were nearly always visible on the shell and were counted as annuli.

A comparison of ages derived by counts of external annuli and by thin sectioning on 82 specimens of *Fusconaia cor* and 49 *Pleurobema oviforme* indicated that counts of external annuli consistently yielded underestimates of ages (Fig. 4). Differences in ages determined by the two methods were highly significant ($P < 0.01$). The degree of underestimation was directly proportional to age estimates; the older the specimen, the greater the underestimate of age by the growth ring method. The two methods yielded similar ages for *F. cor* up to age 10, but mussels 11 to 25 years old were underestimated by 1 to 5 years when external annuli were counted. Thin-sectioning was more effective, particularly on old specimens (> 20 yr). Eight valves of *P. oviforme* older than 20 years could not be aged externally due to periostracum damage; these specimens ranged in age from 25 to 56 years based on thin-sections.

On thin-sections of the latter two species, marks were made adjacent to the exit location of each annulus at the shell margin to allow visual comparisons with cross-sectioned shells from which the thin-sections were cut. Comparison of the two clearly corroborated the occurrence of one externally visible annulus with its internal counterpart in every shell. This external-internal comparison also demonstrated the occasional presence of thinner, false annuli on the shell surface that had no counterpart internally. Generally, internal annuli were much easier to distinguish than external annuli, especially near the shell margin of older specimens.

DISCUSSION

Deposition of one prominent growth band annually was validated in 12% of the tagged specimens that were recovered from the three study streams. The relatively low recovery rate (36%) and slow growth (< 1 mm) of most specimens limited the availability of a larger sample size. Negus (1966) recovered only 56 (9.7%) of 572 marked specimens of three freshwater mussel species in the Thames River, England after 1 year to validate annulus formation; of these, 43 (77%) showed an annulus. Although recovery rates of marked bivalves have been typically low in both freshwater and marine environments (Murawski *et al.*, 1982; Schaul and Goodwin, 1982), formation of annual growth bands in bivalves from temperate climates appears to be common. In the tropics, unionids also

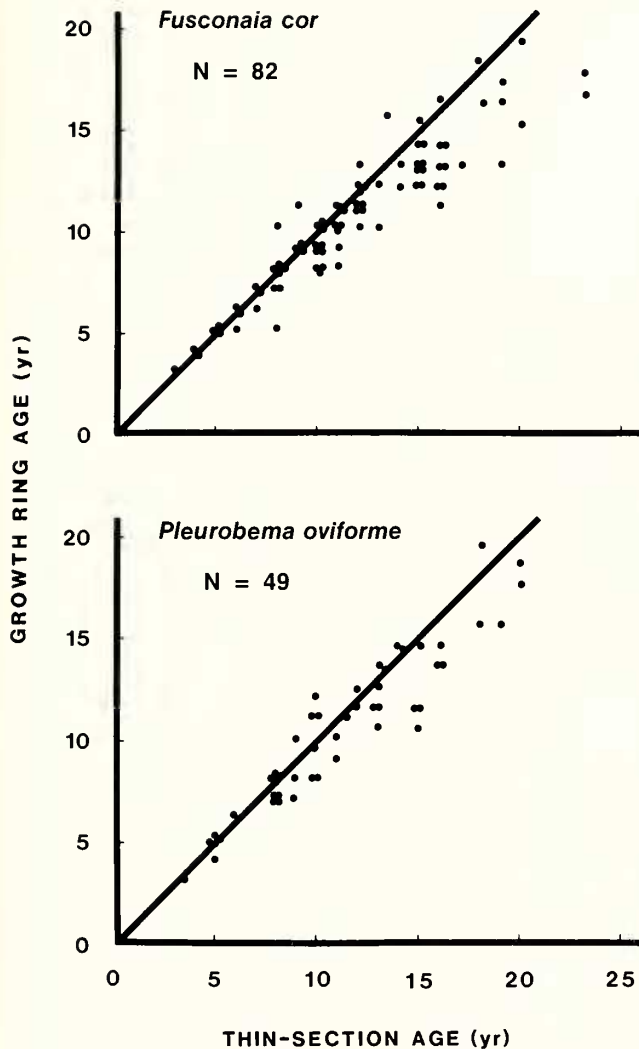


Fig. 4. A comparison of age estimates for two species aged by the thin-sectioning and external growth ring methods. Data points below the 45° line represent underestimates of specimen ages by the growth ring method.

exhibit shell bands, but the causes for their formation are probably different from those for temperate species (McMichael, 1952). This apparent regularity in banding could lead some investigators to assume that annulus formation is a universal phenomenon and that age validation might not be necessary. However, we caution that annual periodicity of growth line deposition is a hypothesis that should be confirmed for each species and locality before it is accepted.

The slow growth of most tagged specimens (96% grew less than 2 mm per year) was the major handicap in age validation. Growth increments along the shell margin of these specimens were insufficient to allow clear separation of growth during the year after tagging from growth in the penultimate year. Ages of most of the tagged specimens, determined later by thin-sections, were 8 to 20 years. These older, larger

specimens proved to be unsuitable, in retrospect, for this component of the study. Our age validation efforts were most successful with mussels of the relatively faster growing, younger age-classes. Therefore, a range of size classes of sufficient number should be used in age validation to overcome the difficulties posed by the slow growth of adults of riverine species.

Other problems associated with slow growth included accuracy of caliper measurements and growth layer detachment. Unnotched mussels that grew less than 1 mm per year had to be excluded because rough shell margins contributed to measurement error with calipers, and annulus deposition could not be confidently ascertained. The narrow growth band along the shell margin often became brittle after the specimens were killed and occasionally broke during measurement or thin-sectioning. Despite these problems with age validation, successes and failures provided experience that improved precision in age determinations of shells. For shells that grew sufficiently for measurement during the 1 year period, the formation of a single growth band per year was confirmed. The identification of both internal and external growth bands for a specimen facilitated the recognition of true versus false annuli and contributed to our confidence in age determinations.

As judged by counts of annuli on mussel shells and growth measured for up to 4 years at study sites, adults of riverine species in Virginia grow slowly and reach maximum ages greater than those reported for lentic species (Grier, 1938; Stansbery, 1961). Longevities of the species aged by thin-sections ranged from 22 to 56 years. These ages exceed those reported for some species in the Mississippi River (Coon *et al.*, 1977), are less than the extreme age (> 100 yr) reported for *Margaritifera margaritifera* L. in Europe (Hendelberg, 1960), but are apparently similar to ages of other slow-growing species (Isley, 1914; Stansbery, 1971). Isley (1914) and Coker *et al.* (1921) reported that light-shelled species grow rapidly, and subsequent studies on *Anodonta* spp. and other thin-shelled species have confirmed their observations (Stansbery, 1961; Negus, 1966; Haukioja and Hakala, 1978). In comparison, they noted that growth in length of heavy-shelled species is most rapid in early life but slows considerably, such that growth lines become tightly spaced and difficult to differentiate. Coker *et al.* (1921) computed mean growth rates of roughly 6 mm/yr for medium-sized individuals of thick-shelled species (*Quadrula* spp.), and Isley (1914) observed shell growth to be roughly 1 mm/yr for older (larger), riverine individuals. Riverine populations of at least some mussel species therefore contain many older, slow-growing cohorts. Based on the slow growth, closely spaced annuli, and considerable longevity of mussels, it is imperative that specimens be accurately aged if exploitation potential or population statistics are to be assessed from age-class structure and abundance (Moyer, 1984).

Although the formation of growth bands is the key process that allows age determination, it is not well understood. Band patterns on freshwater mussel shells occur in two varieties, wide, dark bands at fairly regular intervals, and lighter bands that are irregularly spaced (Tevesz and Carter, 1980). The mechanism through which these bands are incor-

porated into the mussel shell is still unclear. Explanations for this mechanism have been put forth by several authors, and were reviewed by Lutz and Rhoads (1980), Tevesz and Carter (1980), and Day (1984). According to the hypothesis advanced by Lutz and Rhoads (1977) from research on marine molluscs, under conditions favorable to growth, bivalves add to their shells by the deposition of successive laminae of calcium carbonate and conchiolin, an organic-rich substance secreted by the mantle. Periods unfavorable for growth, such as winter in temperate regions, apparently produce changes associated with anaerobic metabolism that lead to the deposition of a thin, dark, organic-rich growth band in the valves. Conversely, the hypothesis presented by Coker *et al.* (1921) and summarized by Tevesz and Carter (1980) was developed through research on freshwater mussels. This hypothesis describes the "doubling-up" of shell layers resulting from mantle retraction and re-extension which produces the visible appearance of a dark ring on the shell. Hence, dark annual rings would be produced by the frequent "doubling-up" of the shell along growth edges produced by frequent growth interruptions from the onset or outset of cold weather (winter). Either of these hypotheses could explain the prominent annual rings that we observed, formed in winter and visible by late spring in Virginia.

There was no indication of long-term tagging or marking stress on shell growth of species recovered for age validation. Unmarked, freshly dead specimens and shells from muskrat middens showed growth increments and rates similar to those in tagged and marked shells of comparable ages (Moyer, 1984). Brousseau (1979) also reported no significant differences in growth rate between handled and unhandled softshell clams (*Mya arenaria* L.) Handling stress was reported in earlier studies with freshwater mussels (Isley, 1914; Coker *et al.*, 1921; Negus, 1966), and notching of bivalves can result in the formation of disturbance lines in shells (Lutz and Rhoads, 1980). Our handling and marking procedures probably resulted in some stress of mussels, and disturbance lines were formed on many specimens that we marked and later examined. These lines were less prominent than annuli and apparently were formed at the time of marking. However, there was no evidence, based on mussel behavior after marking in the laboratory and comparative growth between marked and unmarked specimens, that the stress was more than temporary.

Shell ashing and acetate peels, by the methods described, proved to be ineffective techniques for use on freshwater mussels. However, the combination of 5% HCl etching solution and 15-45 sec etching time provided some peels of suitable quality. Recent modifications and improvements in the acetate peel technique could now make this method more applicable to freshwater bivalves (Ropes, 1987), and further testing is warranted.

Thin-sectioning of shells was judged to be the most consistent and accurate technique for age determinations. Thin-sections provided the highest degree of resolution for all species examined, and for all sizes and ages, from 15 to 210 mm and 3 to 56 years. Annulus formation was readily apparent in cross-sections of marked shells, and true and false

annuli could be easily separated. Minor shortcomings of the thin-sectioning technique were the 0.5 to 1 hr required to prepare a specimen for examination, the need for several cuts on large shells to fit the petrographic slides (27 x 46 mm) used in this study, and the difficulty in sectioning small shells (< 20 mm). Because small, thin shells often were too brittle to withstand the pressure of the cutting blade or chuck used to hold the shell in place, we suggest that bioplastics be used for embedding the shells. Modification of the equipment or technique should overcome these minor problems.

We observed occasional inclusion of small particles of sediment in shells, which produced the formation of a thick, dark line internally and externally, especially on female *Villosa vanuxemi*, as noted previously. This band was a false annulus because it was incomplete and usually occurred only in the vicinity of the foreign particle. Its formation is perhaps evidence of the adventitious conchiolin layering reported by Beedham (1965) and reviewed by Tevesz and Carter (1980). Such layers are described as being a conchiolin-rich damage response mechanism, often found in unionids having thin-shelled umbonal areas. They apparently are produced to mitigate damage caused by extraneous water, sediment, or other material entering through an abnormal separation between the mantle and shell margin.

Our test of the growth ring method confirmed the inadequacy of this technique, as previously noted by Rhoads and Lutz (1980). Erosion and corrosion of shells, separation of true from false annuli, and difficulty in counting closely deposited growth bands in older shells produced consistent underestimates of specimen ages. These errors in age, even on shells with relatively clear annuli such as those of *Fusconaia cor* and *Pleurobema oviforme*, would undoubtedly occur with most other unionids and result in erroneous ages and, consequently, imprecise population statistics. Jones *et al.* (1978) cautioned that growth curves based on external growth lines probably underestimate growth rate in young clams and overestimate it in old ones. Our results with freshwater mussel shells support this conclusion and indicate that the growth ring method provides only an estimate of mussel ages at best, particularly for older cohorts. With the current availability of sectioning techniques to provide more accurate ages of unionids, we recommend that use of the growth ring method be discontinued for all but the younger age classes or rapidly growing species that are age-validated.

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